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Isolation and Identification of Cellulose Degrading Bacteria from Municipal Waste and Their Screening for Potential Antimicrobial Activity

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Abstract: Municipal Solid Waste (MSW) is a rich source of lingo-cellulosic materials thus providing an intense environment for the growth of cellulolytic bacteria. The present study was conducted to isolate and identify cellulose degrading bacteria from MSW dumped in different localities of Peshawar and its vicinity and their screening for potential antimicrobial activity. The cellulolytic bacteria were screened out and isolated from the collected samples by serial dilution method on modified Czapeck (CMC) agar medium and subsequent Congo red assay. The total 108 isolates obtained were further analyzed for cellulose degrading activity qualitatively through Congo red assay. Out of 108 isolates, only 15 isolates were selected on the basis of cellulose hydrolyzing activity (zone ratio 2.5 and above). The antimicrobial activity of these isolates was also determined against different bacterial and fungal human pathogens. These isolates were identified on the basis of standard biochemical tests in Bergey's manual. Among the 15 isolates one belonged to *Pseudomonas spp.*, one to *Aeromonas spp*., one to Pasteurella spp., two belonged to *Staphylococcus spp*. and ten to *Bacillus* genus. Among them, *Bacillus spp.* SD F has shown a remarkable ability of cellulose hydrolysis in terms of both Congo red assay by giving a zone ratio of 3.4 and enzyme activity of 0.2514 $IUmL^{-1}$. The isolates showed antibacterial activity against one or the other among the entire selected target organisms except for *P. aeruginosa*. *Bacillus species* showed antibacterial activity from moderate to high against the pathogens where as *Staphylococcus species* and *Pestuerella pneumotropica* showed moderate activity. The antifungal activity against the target pathogens was shown by the isolates of *Staphylococcus spp.* and some *Bacillus species.* Isolate PI 2 (*Bacillus sp.*) showed high activity against *A. niger* by giving a zone of inhibition of 17mm while isolate 36 F (Aeromonas salmonicida) showed high antifungal activity against *C. albicans* by giving a clear zone of 16 mm. The results of the present study may provide the basis for the utilization of cellulose as an energy source for such bacteria having the ability to produce therapeutic agents by utilizing a less expensive carbon source.

Key words: Cellulolytic Bacterial Species · Carboxymethyl Cellulose · Cellulose · Glucose · Antibacterial Activity · Antifungal Activity

INTRODUCTION

The phenomenal increase in world's population and accelerated pace of urbanization has lead to the increased production of waste which renders the problem of management of municipal waste throughout the world especially in Asian countries [1]. About 43% of the world's municipal waste is generated annually by Asian countries while the North American countries and European Union contributes to 28% of it [2]. Several measures have been taken to tackle this problem. Among the various options available, the most modern and

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appropriate one is the recycling of this municipal waste in a natural way [1-3]. MSW?? is rich in Lignicellulosic material mainly composed of cellulose and lignin with a small ratio of hemicelluloses [4].

Lignicellulosic materials can be turned into a worthwhile and effective asset by utilizing microorganisms which can use it as a sole carbon source and results in the production of valuable substances such as different organic acids and antibiotics [5]. But unfortunately most of this waste is burnt off for disposal throughout the world [6].

The municipal waste is rich in cellulose, which is a cheap, abundant bio polymer and renewable energy source. It is a polysaccharide having a fibrous crystalline appearance made up of the repeating units of D-glucose linked by β-1, 4- glucosidic linkage. It is a water soluble compound and has a high molecular weight [1, 4]. For cellulolysis, a set of cellulases enzymes is required including exoglucanases, endoglucanases and β-glucosidases, which acts in a synergistic manner [7].

Microorganisms having the ability to degrade cellulosic compounds are of great importance from different biological and ecological point of view. The cellulose degrading ability of fungal and bacterial species has provided a broad platform for research in determining the physico-biochemical properties of these microorganisms as well as their use in different biotechnological processes [8]. Fungal species hold a promising position in producing antibiotics and in utilizing cellulose as a carbon source and a lot of work has been done on it [9, 10]. By far the most extensively studied fungi are the soft-rot fungi such as *Trichoderma viride* and *Trichoderma reesei.* In last few years, the enzymology of this cellulose has been well demonstrated. The *P. chrysosporium* has been identified to secrete two different enzymes one cell bound while the other extracellular [11-14]. Gilligan and Reese [15] were the first to examine this synergy of cellulolytic enzymes in the fungal specie *Trichoderma viride*.

While in case of bacteria, studies have been done on Lignicellulosic degradation by different strains such as *Cellulomonas, Pseudomonas* and *Actinomycetes* species which hydrolyze lignin as well as the carbohydrate portion of the waste [16]. Cellulolytic microorganisms especially bacteria utilize this waste for energy source by reducing cellulose and results in the production of economically beneficial compounds, such as monomeric sugars, antibiotics, single cell proteins. A lot of emphasis and research has been done on cellulolytic fungal species as compared to bacterial cellulose degrading species, although bacteria exhibit a high rate of growth. However, the application of efficient cellulolytic bacteria for the reduction of cellulose is still a novel approach. Bacterial cellulases usually act as a highly effective and potent catalyst [17].

Different Fungal and bacterial species are known for the production of certain secondary metabolites which help them to survive in an inimical and competitive environment. Antibiotic production is a normal phenomenon for the soil dwelling microorganisms. They secrete a broad range of antibiotics which helps in their defense mechanisms [18]. The use of fungal and bacterial species for antibiotic production has been preferred over the conventional use of plants and animals due to their ability to generate desirable results in an appreciable manner [3].

Pakistan being a third world country suffers from the very same problem of Waste management. The main objective of this research study was the isolation and identification of those bacterial species which are capable of efficiently hydrolyzing the cellulosic compounds in the municipal waste and are potent enough to produce antimicrobial agents which can be used for the antibiotic production and can act as enhancer in the biodegradation process of these Lignicellulosic compounds.

MATERIALS AND METHODS

Sample Size and Location: A Total of 70 MSW soil samples were collected from different localities of Peshawar and its vicinity including Reggi (11), Gulbahar (12), Kohat Road (11), Badaber (10), Tehkal (10), Station Chowk (06) and Ring Road (10).

Sample Collection: For each sample, soil was first dug out with clean shovel up to 15 cm depth by using disposable and sterile wooden plough. Soil was then mixed thoroughly and foreign materials like pebbles, polythene bags and stones were removed. Approx 10 g was transferred to sterile zip lock bag. All the soil samples after collection were properly sealed, labeled and sent to laboratory where they were kept at 4°C.

Screening, Isolation and Purification: Screening and isolation of cellulolytic bacterial species was done on modified Czapeck agar medium (CMC medium), by serial dilution and direct plating method. 1 g of soil sample was added in 9 mL of saline and was shaked thoroughly. Further dilutions were made from it up to 10^{-5}. After dilutions, 1 mL inoculum of serially diluted samples (10^{-5}

concentration) was spread on CMC agar media plates and was incubated at 37°C for 2 days. After incubation, media plates were flooded with 0.2 % (w/v) Congo red dye for 20 minutes (Congo red assay). Dye was discarded and plates were flooded with 1 M NaCl for 20 minutes. NaCl was discarded. Colonies were marked and were subcultured on CMC media [19].

Detection of Cellulase Producing Activity of Bacterial Species

Congo Red Assay for Cellulase Producing Activity of Bacterial Isolates: For zones determination, cultures were grown in circular batches on CMC agar media plates and were flooded with Congo red dye. The cellulose hydrolyzing activity was measured by the appearance of clear zones around the culture batches. The isolates with the highest zone diameter to colony diameter were selected for quantitative analysis of cellulase enzyme and antimicrobial activity.

Carboxymethyl Cellulase (Cmcase) Assay of Bacterial Isolates: Quantitative analysis of cellulase production was done by CMCase assay for Endo- β-1, 4-glucanase. For this purpose, DNS method was used for reducing sugar estimation [20].

Identification of Bacterial Isolates: Bacterial isolates were identified by using standard identification tests given in Bergey's Manual of Determinative Bacteriology [21].

Detection of Antimicrobial Activity: Antimicrobial activity of these cellulolytic isolates was determined by well diffusion method against different bacterial and fungal human pathogens [22].

Antibacterial Activity of Cellulolytic Bacteria: Antimicrobial activity of these isolates was determined against five different bacterial human pathogens *Staphylococcus aureus* ATCC 29213*, Escherichia coli* 7*, Klebsiella pneumoniae* ATCC 700603*, Pseudomonas aeruginosa*?? and *Enterococcus faecalis* ATCC 29212 on fresh Nutrient agar media at 37°C against a positive control (Streptomycin 1mg/mL). Wells of 0.5 cm were made with the help of a sterile borer on the media and 100 uL of the supernatant and streptomycin was poured to the respective wells while for negative control sterile distilled water was used. Then zones of inhibition for the supernatant and positive control were measured. Each experiment was run in duplicate.

Antifungal Activity of Cellulolytic Bacteria: The antifungal activity of these isolates was tested against pathogenic fungi e.g. *Candida albicans* and *Aspergillus niger* on fresh Potato Dextrose Agar (PDA) plates and were incubated at 25-27 °C for 5 days. A sterile cotton swab was used to uniformly spread the fungal culture on PDA plates. The plates were allowed to dry for 15 minutes. Wells of 0.5 cm were made with the help of a sterile borer on the media and 100 uL of the supernatent and Fluconazole (Concentration 1mg/mL) was poured to the respective wells while for negative control sterile distilled water was used. Then zones of inhibition for the supernatant and positive control were measured. The experiment was repeated twice [23].

RESULTS AND DISCUSSION

Screening, Isolation and Purification of Cellulolytic Bacterial Species: Screening and isolation procedure resulted in 158 cellulolytic bacterial isolates from different MSW samples which on further sub culturing on Czapeck modified agar (CMC) medium gave 108 pure isolates.

Congo Red Assay for Cellulase Production: All the 108 bacterial isolates showed clear zones around colonies. Out of these, only 15 bacterial isolates showed zone to colony ratio in the range of 2.5 and above. Among these 2 bacterial species showed significant cellulolytic activity in the range of 4.1-5.5 ratios, 4 showed high activities in a range of 3.1- 4.0 while 9 isolates showed moderate cellulolytic activity in the range of 2.1-3.0 ratios as given in Table 1. These 15 Cellulolytic isolates were selected for further quantitative analysis based on their relatively higher zone ratio as given in Table 2. They were considered to be efficient isolates.

Among the efficient isolates, maximum cellulolytic zone diameter to colony diameter ratio measured was 5.33 while minimum cellulolytic zone was 1.0. These results indicated that the isolated Bacterial species have moderate to significant ability to produce cellulases.

Biochemical Identification of Bacterial Isolates: Different bacterial identification methods were used, which showed that the bacterial isolates were Gram positive rods, Gram positive cocci and Gram negative rods. Out of these 15 isolates, 10 isolates were Gram positive rods that turned out to be *Bacillus species*, while 2 were Gram positive cocci belonging to *Staphylococcus.* The rest 3 isolates were gram negative rods belonging to *Pseudomonas, Aeromonas and Pesturella species*.

Table 1: *Activity ranges of cellulolytic bacterial isolates (Z/C Ratio)*

Cellulolytic bacteria Ratio > 2.5	Moderate cellulolytic activity Ratio 2.1-3	High cellulolytic activity Ratio 3.1-4	cellulolytic activity Ratio 4.1-5.5
15	9	5	2

Table 2*: Efficient cellulolytic bacterial isolates (ratio > 2.5)*

S. No	Cellulolytic isolates codes*	Mean Zone diameter (mm)	Mean Colony diameter (mm)	Ratio Zd/Cd
1	30 H	11mm	4mm	2.75
2	1 C	16mm	3mm	5.33
3	P H2	19mm	4mm	4.75
4	P I2	19mm	7mm	2.7
5	P J2	14mm	4mm	3.5
6	P L2	20mm	7mm	2.86
7	P L1	18mm	7mm	2.57
8	SD C	40mm	15mm	2.66
9	SD E	18mm	6mm	3.0
10	SD F	17mm	5mm	3.4
11	SR H	6mm	2mm	3.0
12	SR I	7mm	2mm	3.5
13	BB H	16mm	6mm	2.6
14	35 C	10mm	4mm	2.5
15	36 F	40mm	13mm	3.08

Note: mm=millimeters, ZD= zone diameter, CD= colony diameter

**The code given to each isolate is derived from the source from which it has been isolated.*

Table 3: *Absorbance, Glucose concentration and Enzyme units of 15 test samples by spectroscopy*

S.NO	Isolate code	Zone ratio* of efficient Cellulolytic bacterial isolates	Glucose concentration mg/0.5 mL* (y=1.083x+0.017) R^2=0.998	Enzyme units (IU ml^{-1})**
1	30 H	0.028	0.0102	0.0038
2	1 C	0.042	0.0231	0.0086
3	P H2	0.064	0.0434	0.0161
4	P I2	0.069	0.0480	0.0178
5	P J2	0.078	0.0563	0.0208
6	P L2	0.030	0.0120	0.0044
7	P L1	0.044	0.0249	0.0092
8	SD C	0.092	0.0693	0.0256
9	SD E	0.035	0.0166	0.0062
10	SD F	0.753	0.6796	0.2514
11	SR H	0.031	0.0129	0.0048
12	SR I	0.506	0.4515	0.1671
13	35 C	0.486	0.4331	0.1602
14	BB H	0.109	0.0850	0.0314
15	36 F	0.029	0.0111	0.0041

**given values are mean of duplicate value*

***formula for calculating CMC unit =mg glucose released * 0.37*

*1.0 mg glucose=1.0/0.18 * 0.5 * 30 u mol/min/m L substrate cleavage*

According to the research study by Lynd *et al*. [24] both *Bacillus* and *Pseudomonas species* are capable of producing cellulases. These both bacterial isolates have firmly cell bound cellulases.

Carboxymethyl Cellulase Assay of Bacterial Isolates: Bacterial isolates processed for quantitative analysis produced different levels of glucose concentration in mg/0.5 mL which were determined from glucose standard curve. These glucose concentrations were translated into enzyme concentration in IU [20].

All these isolates displayed activity in the range of 0.0038 to 0.2514 IU ml^{-1}. Out of 15 isolates processed, maximum amount of CMCase enzyme was released by isolate SD F as determined to be 0.2514 IU ml^{-1} while minimum amount was released as 0.0038 IU ml^{-1} by isolate 30 H as given in the Table 3. Among the remaining isolates some displayed moderate cellulolytic activities while some showed considerably low activity in this method. By comparing the qualitative and quantitative results of cellulolytic activity of all the efficient isolates, it was found that SD F isolate

Table 4: *Isolates showing antibacterial activity*

S.No.	Test Samples	*S. aureus* ATCC 29213 Mean Zone Diameter Value (mm)	*E. coli 7* Mean Zone Diameter Value (mm)	*K. pneumoniae* ATCC 700603 Mean Zone Diameter Value (mm)	P. aeruginosa Mean Zone Diameter Value (mm)	E. faecalis ATCC 29212 Mean Zone Diameter Value (mm)
	Positive control (streptomycin 1mg/ml)	22 ± 1.0	18 ± 1.0	20 ± 1.0	25 ± 3.0	25 ± 3.0
1	*Staphylococcus spp.* 30 H	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	17 ± 1.0
2	*Bacillus spp.* 1 C	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18 ± 2.0
3	*Bacillus spp.* PH 2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	*Bacillus spp.* PI 2	0.0 ± 0.0	10 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	*Bacillus spp.*PJ 2	7.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12 ± 1.0
6	*Bacillus spp.* PL 2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
7	*Bacillus spp.*PL 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	*Bacillus spp.*SD C	0.0 ± 0.0	11 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
9	*Bacillus spp.*SD E	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10	*Bacillus spp.*SD F	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12 ± 1.0
11	*Staphylococcus spp.*SR H	9.0 ± 1.0	0.0 ± 0.0	15 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
12	*Bacillus spp.* SR 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13 ± 3.0
13	Pasteurella pneumotropica 35C	7.0 ± 1.0	0.0 ± 0.0	17 ± 02	0.0 ± 0.0	11 ± 1.0
14	*Pseudomonas aeruginosa* BB H	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15.	Aeromonas salmonicida 36F	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Note: mm= millimeters

Table 5: *Isolates showing anti fungal activity*

S.NO.	Isolate code	*Candida albicans* Mean Zone Diameter Value (mm)	*Aespergillus niger* Mean Zone Diameter Value (mm)
	Positive control (fluconozal 1mg/ml)	18 ± 02	20 ± 01
1	*Staphylococcus spp.* 30 H	16 ± 01	0.0 ± 0.0
2	*Bacillus spp.* 1 C	12 ± 02	0.0 ± 0.0
3	*Bacillus spp.* PH 2	0.0 ± 0.0	17 ± 01
4	*Bacillus spp.* PI 2	0.0 ± 0.0	0.0 ± 0.0
5	*Bacillus spp.*PJ 2	0.0 ± 0.0	0.0 ± 0.0
6	*Bacillus spp.* PL 2	0.0 ± 0.0	0.0 ± 0.0
7	*Bacillus spp.*PL 1	0.0 ± 0.0	0.0 ± 0.0
8	*Bacillus spp.*SD C	0.0 ± 0.0	0.0 ± 0.0
9	*Bacillus spp.*SD E	0.0 ± 0.0	15 ± 02
10	*Bacillus spp.*SD F	0.0 ± 0.0	0.0 ± 0.0
11	*Staphylococcus spp.*SR H	0.0 ± 0.0	0.0 ± 0.0
12	*Bacillus spp.* SR 1	0.0 ± 0.0	0.0 ± 0.0
13	Pasteurella pneumotropica 35C	0.0 ± 0.0	0.0 ± 0.0
14	*Pseudomonas aeruginosa* BB H	0.0 ± 0.0	0.0 ± 0.0
15	Aeromonas salmonicida 36F	0.0 ± 0.0	0.0 ± 0.0

Note: mm= millimeters

(*Bacillus* sp.) had displayed high zone ratio in Congo red assay and maximum amount of enzyme in DNS method.

In contrast to this result of SD F isolate, isolate 30 H had displayed moderate zone ratio in qualitative assay while showed minimum amount of enzyme in quantitative assay. Besides these isolates, isolate 1 C showed maximum qualitative activity by giving the maximum ratio (zd/cd) of 5.33 but it showed enzyme activity among the lowest values i.e. 0.00855 IU mL^{-1}. There are few more isolates which showed such contradictory results with respect to their qualitative and quantitative CMCase activity such as PH 2, SD E and 36 F. These variation in results may be due to fluctuations in experimental parameters such as pH changes in broth medium, incubation time and temperature because of which the bacteria was not able to release enzyme properly [24].

A study report by Krootdilganandh [25] showed that out of 77 thermo tolerant bacteria grown on CMC agar, an isolate CMV4-4 showed the highest enzymatic activity. However this isolate showed the least clearing zone during qualitative analysis.

Antibacterial Activity: For S*taphylococcus aureus* ATCC 29213, Out of the 15 isolates only 3 isolates SR H, 35 C and PJ 2 showed low activity while the rest of the isolates

were inactive (Table 4). The maximum zone i.e. 9.0 mm was given by SR H while 35 C and PJ 2 showed the clear zone of 7.0 mm. SR H, *staphylococcus species*, has shown moderate activity against *S. aureus*. This can be due to the inherit defense mechanism of a strain to secret antimicrobial agents such as bacteriocins that prevent the growth of other strains of the same specie [18]. Gram positive bacteria especially certain species of staphylococcus are also known to exhibit ability to produce such antimicrobial agents like colicin by *E. coli* against other strains such as ColN and ColE1 against E. coli F4 (K88) [26, 27]. For *E. coli* 7, the isolates which showed activity were SD C and PI 2 by forming a clear zone of 11 mm and 10 mm respectively. For K. pnumoniae ATCC 700603, SRH and 35 C were the only to show high activity by giving zone of inhibition of 15 mm and 17 mm respectively. None of the 15 isolates showed any kind of activity against *P aeruginosa*. For *E. fecalis* ATCC 29212, out of the 15 isolates only 6 isolates showed activity. The highest zone of inhibition was given by 1 C i.e. 18mm while 35 C gave the lowest zone of 11 mm.

Antifungal Activity: Out of 15 isolates only two isolates showed activity against each of the target fungal pathogens (Table 5). Against *A. niger,* isolate PH 2 showed high activity by forming a zone of inhibition of 17 mm while SD E showed antifungal activity by forming a clear zone of 15 mm. While for *C. albicans*, isolate 30H showed high antifungal activity by forming a clear zone of 16 mm where as isolate 1C showed moderate activity by giving a zone of inhibition of 12mm.

This research work concluded that bacterial species isolated from different locations of Peshawar showed the remarkable ability to degrade Carboxymethyl Cellulose in the media. They also showed their abilities to produce antibacterial and antifungal metabolites against pathogenic bacterial and fungal species. However the cellulase production was low to high indicating that these species have a variable ability to degrade CMC meaning thereby these bacterial isolates can be used for the production of therapeutic agents as well as can be used as inoculants for enhancing the degradation process of cellulose.

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